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Intra-Arterial Delivery of AAV Vectors to the Mouse Brain After Mannitol Mediated Blood Brain Barrier Disruption

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Abstract

The delivery of therapeutics to neural tissue is greatly hindered by the blood brain barrier (BBB). Direct local delivery via diffusive release from degradable implants or direct intra-cerebral injection can bypass the BBB and obtain high concentrations of the therapeutic in the targeted tissue, however the total volume of tissue that can be treated using these techniques is limited. One treatment modality that can potentially access large volumes of neural tissue in a single treatment is intra-arterial (IA) injection after osmotic blood brain barrier disruption. In this technique, the therapeutic of interest is injected directly into the arteries that feed the target tissue after the blood brain barrier has been disrupted by exposure to a hyperosmolar mannitol solution, permitting the transluminal transport of the therapy. In this work we used contrast enhanced magnetic resonance imaging (MRI) studies of IA injections in mice to establish parameters that allow for extensive and reproducible BBB disruption. We found that the volume but not the flow rate of the mannitol injection has a significant effect on the degree of disruption. To determine whether the degree of disruption we observed with this method was sufficient for delivery of nanoscale therapeutics, we performed IA injections of an adeno-associated viral vector containing the CLN2 gene (AAVrh.10CLN2), which is mutated in the lysosomal storage disorder Late Infantile Neuronal Ceroid Lipofuscinosis (LINCL). We demonstrated that IA injection of AAVrh.10CLN2 after BBB disruption can achieve widespread transgene production in the mouse brain after a single

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administration. Further, we showed that there exists a minimum threshold of BBB disruption necessary to permit the AAV.rh10 vector to pass into the brain parenchyma from the vascular system. These results suggest that IA administration may be used to obtain widespread delivery of nanoscale therapeutics throughout the murine brain after a single administration.

Keywords

Intra-arterial; neural drug delivery; MRI; blood brain barrier; adeno-associated virus

Introduction

The brain is a notoriously difficult organ to access therapeutically due to the presence of the blood brain barrier (BBB). The BBB is a complex physical barrier of specialized endothelial and astrocytic cells that selectively prevents many molecules from passing from the blood to the brain parenchyma [1]. Since many drugs do not cross the BBB efficiently, achieving therapeutic concentrations of drug in the target tissue after systemic delivery can often require toxic doses, and many promising therapies for disorders of the central nervous system (CNS) fail due to inadequate delivery.

Direct delivery of therapy to the CNS through intra-parenchymal injection or controlled release implants (thereby physically bypassing the BBB) can obtain high local concentrations of the delivered agent, but these techniques are limited by the spatial distribution that can be obtained and require opening of the skull and meninges.

One technique that offers the potential to access large volumes of brain without performing open brain surgery is intra-arterial (IA) injection. In this technique a solution containing the agent of interest is injected directly into the arteries that supply the target tissue through an endovascular catheter [2–7]. However, IA delivery to the CNS is still limited by the BBB. The most commonly used method of permeabilizing the barrier to allow transluminal transport of injected agents is mannitol mediated osmotic disruption which has been widely used in preclinical and clinical studies [3, 4, 8–10]. To date, most preclinical studies have been performed in larger animals due to the difficulty associated with surgical positioning of the endovascular catheter and as a result little data exists on IA injection in mice.

The large number of murine genetic models of CNS disorders would make the ability to reproducibly perform successful IA injections in mice of great value to the drug development community. Our goal in this work was to establish the injection parameters for IA delivery of therapeutic constructs to the brain after mannitol mediated BBB disruption in the mouse. We first examined the effects of IA mannitol injection flow rate and volume on the degree of BBB disruption using dynamic contrast enhanced magnetic resonance imaging (DCEMRI). We surgically implanted custom microcatheters in the internal carotid artery (ICA) of anesthetized mice and performed IA injections of the paramagnetic MRI contrast agent gadopentate dimeglumine (Gd-DTPA) while continually acquiring MR images two minutes after disrupting the BBB with an IA injection of 25% (wt/v) mannitol solution.

Next we applied these findings to the *in vivo* IA delivery of a rhesus macaque derived adeno-associated viral (AAV) vector to the brain to determine if the degree of disruption we observed would be sufficient for delivery of a relatively large nano-scale therapeutic construct. We performed IA injections of AAVrh.10CLN2, an AAV vector encoding the human CLN2 gene which is the gene of interest in Late Infantile Neuronal Ceroid Lipofuscinosis (LINCL). The systemic nature of LINCL makes it an attractive potential target for IA delivery.

We found that the volume of the mannitol injection but not the flow rate had a significant effect on the degree of BBB disruption. Further we demonstrate that at high mannitol doses the BBB disruption is sufficient to allow transvascular delivery of AAV as evidenced by high levels of the transgene product which localized to neurons five weeks after treatment. We also show that the degree of BBB disruption seen on DCE-MRI scans is predictive of eventual viral particle deposition within the brain.

Materials & Methods

Endovascular Microcatheters

Custom polyimide endovascular microcatheters were fabricated as previously described [11]. Briefly, 169 μm outer diameter monolumen polyimide tubing (MicroLumen, Tampa, FL) was cut to 10 cm lengths and one end was secured in a polypropylene injection hub (SmallParts Inc.) using two-part epoxy (Miller Stephenson, Danbury, CT). The adhesive was then cured for two hours at 80° C. Next, a 13 cm length of 7-0 monofilament suture was passed into the lumen of the microcatheter to prevent the tubing from being crushed or kinked during handling.

AAVrh.10CLN2 Production

The genome of AAVrh.10CLN2 includes the inverted terminal repeats from AAV2 surrounding the expression cassette [12]. The expression cassette consists of a CMV/ β -actin hybrid promoter [13, 14], the human CLN2cDNA with an optimized Kozak translational initiation signal before the start codon, and a rabbit β -globin poly(A) sequence. The vector was produced by cotransfection of forty 150 mm plates (80% confluent) of 293T cells with 500 μg of an expression cassette plasmid (pAAV2-CAG-CLN2) and 1 mg of an adenovirus/AAVrh.10 helper plasmid (pPAK-MARh.10) using PolyFect reagent (Qiagen Sciences, Germantown, MD). The helper plasmid included the AAV2 *rep* gene and AAVrh.10 *cap* gene necessary for viral reproduction and capsid production.

Cells were harvested (1150 g, 15 min) 72 hours post-transfection and a crude viral lysate (CVL) was made by three cycles of freeze-thawing. Benzoase (50 U/ml; Sigma-Aldrich, St. Louis, MO) was used to remove any contaminant genomic DNA. The remaining CVL was centrifuged at 3,300 g for 20 min and the supernatant applied to a discontinuous iodixanol gradient. It was then purified by Q-HP ion exchange chromatography and centrifugally concentrated into PBS. Vector concentration in particle units was determined by TaqMan real time PCR with absolute quantitation. To confirm functionality, 293-ORF6 cells were

infected with AAVrh.10CLN2, and TPP1 enzymatic activities were assessed in the cell supernatant 72 hours post-infection [13, 15].

All vectors used in these studies were shown to be sterile by growth for 14 days on medium supporting the growth of aerobic bacteria, anaerobic bacteria, or fungi, and were endotoxin free to a level of <200U per milliliter by the Endosafe method (Charles River, Charleston, SC).

Animals and Surgical Catheterizations

Male CD-1 mice (22–24 g; Charles River Laboratories Inc.) were anesthetized via isoflurane inhalation (1–3% in O₂ at 1–2 l/min). A mid-line longitudinal incision was made over the hyoid bone, and the common carotid artery bifurcation was exposed using blunt dissection. The superior thyroid and occipital arteries were permanently ligated, and the external carotid artery (ECA) was dissected from the underlying structures. A permanent tie was placed on the distal ECA, and a temporary ligature of 6–0 monofilament suture was placed on the proximal ECA to obtain a flow free segment of vessel. A sterilized microcatheter was flushed with heparin (1:1000 units per ml (Heparin sodium; Baxter Healthcare Corporation)) and introduced to the lumen of the ECA via a small arteriotomy, advanced retrograde to the proximal ligature, and secured in the ECA using two purse-string suture ties. The distal ECA was then severed, and the catheter was manually rotated so that the tip was oriented cephalad in the ICA distal to the carotid bifurcation [16].

Prior to each injection the hub of the microcatheter was filled with saline to minimize the introduction of air, the guidewire suture was removed from the catheter lumen, and the microcatheter was connected to a syringe mounted on an MRI compatible programmable syringe pump small (PHD 2000, Harvard Apparatus Inc., Holliston MA) using small bore Teflon tubing and Luer-Lock fittings (Idex Health and Science, Oak Harbor, WA).

After the injection, the catheter was carefully removed and the ECA permanently ligated. The incision was irrigated with sterile saline and closed using interrupted sutures. Survival animals were allowed to recover and received regular post-operative health checks and analgesics. Non-survival animals were sacrificed via an overdose of sodium pentobarbital while still deeply anesthetized. All animal experiments were approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee (IACUC).

MRI Based Assessment of IA BBB Disruption

Catheterized mice were moved to the bore of a 7.0 tesla small animal MRI system (BioSpec 70/30 USR, Bruker Biospin, Billerica MA). A 25% wt/vol mannitol solution was injected through the implanted catheter to disrupt the BBB. Four mannitol injection volumes were examined – 125 µl, 250 µl, 500 µl, and 750 µl – each at a constant flow-rate of 1000 µl/min (n = 8 animals per group). The effect of the mannitol flow-rate on BBB disruption was also examined by injecting 750 µl of mannitol at flow-rates of 250 µl/min, 500 µl/min, 800 µl/min, and 1000 µl/min (n = 8 animals per group). Two minutes after the end of the mannitol injection, mice were injected IA with 750 µl of Gd-DTPA (1:19 dilution of Magnevist®, Bayer Healthcare Pharmaceuticals, in saline) at a flow rate of 800 µl/min while monitoring the distributed volume and tissue uptake/washout of the injected agent in real time using a

FLASH gradient echo pulse sequence with TR = 25 ms, TE = 3.8 ms, FOV = 25 mm×25 mm, and a matrix size of 192×256. This allowed us to capture one imaging frame every 3.6 seconds before, during, and after the Gd-DTPA injection. Studies of IA mannitol mediated BBB disruption have found that barrier opening begins immediately upon mannitol administration and that the optimal time for administration of the second injection is at 0–5 minutes post mannitol delivery [17]. For this reason, the second IA injection in our experiments was performed as quickly as could be consistently reproduced following the initial mannitol injection. In this case the delay was two minutes to ensure that there was sufficient time to obtain baseline data prior to the IA injection of Gd-DTPA.

All imaging data was analyzed using ImageJ [18]. Measurement regions-of-interest (ROIs) were placed around each hemisphere, and also in three arterial territories in each hemisphere (AChA, MCA, and ACA territories), and used to generate traces of the absolute signal in each ROI over time. These data were then normalized using the average signal in each ROI of the baseline prior to the Gd-DTPA injection. The mean enhancement over baseline was taken to be the average value of the normalized signal in the appropriate ROI for the last 60 imaging frames captured.

All data are presented as the mean $\pm$ the standard error of the mean. Treatment groups were compared using analysis of variance at the 95% confidence interval and *post hoc* Tukey's tests.

Intra-arterial AAVrh.10CLN2 Injections

Animals that received IA injections of AAVrh.10CLN2 were catheterized as described above, and given an injection of 125 μ l, 250 μ l, 500 μ l, or 750 μ l of 25% wt/vol mannitol solution at a flow rate of 800 μ l/min. After one minute, a solution was injected containing the AAVrh.10CLN2 dose (3.18×10^{10} , 6.35×10^{10} , 1.27×10^{11} , or 2.54×10^{11} gc/mouse) diluted to a final volume of 100 μ l in normal saline at a flow rate of 50 μ l/min. All injection lines were flushed with normal saline to ensure that the full dose was delivered to the ICA.

Immunohistochemistry

TPP1 distribution in the mouse brain was assessed five weeks after gene transfer by both immunohistological (IHC) and immunofluorescent (IF) staining. Animals were sacrificed via transcardiac perfusion with cold PBS and 4% paraformaldehyde 5 weeks post-injection. Brains were harvested, post-fixed in 4% paraformaldehyde for 24 hours, cryoprotected in 30% sucrose solution overnight, and then sagittally sectioned and mounted on slides for staining (Histoserv Inc., Germantown, MD). Slides were prepared for IHC staining by first quenching endogenous peroxidase activity and then blocking both endogenous biotinylation and avidin. The slides were also treated with a Mouse on Mouse (MOM) kit (Vector Labs) because of the use of mouse primary antibodies. The slides were labeled using a primary antibody targeting the TPP1 protein (mouse anti-hTPP1, 1:1,000 dilution). A biotinylated anti-mouse secondary antibody was used, followed by a peroxidase developing solution. The slides were visualized on a wide-field transmitted light microscope.

A simple algorithm was developed for quantifying the results of IHC staining, including conversion of the color images to 8-bit (0–255 scale) format, grayscale inversion, application of a threshold, and generation of a binary image such that all pixels above the threshold were assigned the value unity and those below the threshold the value zero. The threshold was selected to be approximately one standard deviation below the mean value of a series of ROI's where there was unequivocal staining at the highest virus dose. For this work the resulting threshold was set equal to 104. Next, for each slice, a region of interest was drawn around the brain and the number of stained voxels within the region was calculated relative to the total number of voxels. The result was expressed as a percentage of stained voxels in the region and plotted versus the vector dose. The number of voxels in each region of interest was typically around 2 million, and an average of 14 slides (14.1 ± 2.3), were evaluated for each series at a given virus and mannitol dose. The cerebellum and olfactory bulb were excluded from the analysis. This method was used to determine the relative staining efficiency in both virus and mannitol dose escalation studies.

For IF, the slides were first treated with the MOM kit, and then were co-labeled using a primary antibody targeting TPP1 (mouse anti-hTPP1 primary, 1:1,000 dilution) and antibodies against either neurons (mouse anti-NeuN, Chemicon International, Temecula, 1:2,500) or astrocytes (guinea-pig anti-GFAP, Advanced Immunochemical, Long Beach CA, 1:1500). The slides were also counterstained with DAPI for nuclear staining. Secondary antibodies targeting the species of the primary that are were conjugated to either CY-3 or CY-5 were used. Finally, slides were imaged on an epifluorescent microscope, and a composite image of the three different fluorescent images was made.

Biodistribution of TPP1 Activity

IA treated animals underwent BBB disruption as described above via ICA injection of 750 μ l of 25% wt/vol mannitol solution followed 1 minute later by 2.54×10^{11} gc AAVrh.10CLN2 diluted to a final volume of 100 μ l in normal saline at a flow rate of 50 μ l/min. IV treated mice received 750 μ l of 25% wt/vol mannitol solution followed 8 minutes later by 2.54×10^{11} gc AAVrh.10CLN2, both via the tail vein [19]. After 5 weeks, treated mice and treatment naïve control mice (n = 3 per group) were sacrificed by an overdose of sodium pentobarbital and transcardially perfused with cold saline, and the left cerebral hemisphere, heart, lung, spleen, liver, kidney, and quadriceps muscle were harvested for analysis. Tissue extracts were prepared by homogenization in 150 mM NaCl and 1g/L Triton X-100 using a disposable pestle and 1.5 ml matching tube (Kimble-Kontes, Vineland, NJ) and clarified by centrifugation. Supernatants were assessed for TPP1 activity as described previously [20] following the procedure of Sohar *et al* [21].

IV and IA treatment groups were compared for statistical significance using two-tailed Student's t-tests at the 95% confidence interval.

Results

DCE-MRI Studies of mannitol mediated BBB disruption in the mouse

To assess BBB disruption resulting from IA infusion of mannitol, we measured contrast enhanced MRI signals in mouse brain regions accessed by IA delivery as a function of time in regions supplied by the anterior choroidal, middle cerebral, and anterior cerebral arteries (AChA, MCA, ACA respectively) (Fig. 1).

When compared to baseline images, Gd-DTPA introduced into the arteries initially caused a darkening of the vessels due to water T_2 nuclear relaxation time effects [22]. The contrast agent subsequently extravasated into the parenchyma where the reduced concentration resulted in dominant water T_1 relaxation, yielding a signal enhancement plateau. The average value of the signal in the plateau phase relative to baseline was used as a metric for BBB disruption across different mannitol pre-injection conditions (Fig. 2). We tested five different volumes of mannitol injection at a constant flow rate (0 μ l, 125 μ l, 250 μ l, 500 μ l, and 750 μ l) and found that the volume of mannitol injection had a significant effect on BBB disruption in the injected hemisphere, with the greatest disruption achieved with the highest volume of mannitol. Varying the flow rate of the mannitol injection (250 μ l/min, 500 μ l/min, 800 μ l/min, and 1000 μ l/min) did not significantly affect the disruption for a given volume of mannitol preinjection ($n = 8$ animals per group). In addition, distal branches of the cerebrovascular tree were disrupted to a lesser degree than more proximal territories. We also examined possible hydrostatic effects of the IA injection on BBB disruption by performing a saline pre-injection (750 μ l at 800 μ l/min; $n = 5$ animals) in place of mannitol, and observed no significant change in the enhancement levels compared to no pre-injection (data not shown).

IA delivery of AAV after mannitol mediated BBB disruption

Next, we determined whether the extent of BBB disruption we achieved by IA delivery of mannitol was sufficient to allow delivery of nanoscale therapeutics to a large fraction of the brain. Based on the results of our MRI studies of BBB disruption, we chose to use a mannitol dose of 750 μ l delivered at 800 μ l/min as this was sufficient to obtain extensive disruption in the whole ipsilateral hemisphere that is supplied by the anterior circulation of the ICA. We performed a vector dose escalation study by injecting the ICA of mice ($n = 3$ animals per group) with increasing AAVrh.10CLN2 doses one minute after maximal BBB disruption dose (from our DCE-MRI studies). The viral doses used were 3.18×10^{10} , 6.35×10^{10} , 1.27×10^{11} , and 2.54×10^{11} genome copies (gc) per animal. After 5 weeks, mice were sacrificed and TPP1 expression was evaluated using immunohistochemistry. Whole-hemisphere sagittal sections of treated mice exhibited extensive TPP1 expression over the entire anterior circulation that was perfused by the ICA, with most of the staining concentrated in the deep brain structures that are supplied via the AChA. It was also clear that the degree of expression increased with increasing virus dose (Fig. 3a – 3d). At lower virus doses, the transgene expression was concentrated in the more proximal territories of the cerebrovasculature. This was possibly due to the existence of a minimum volume of virus solution needed to adequately fill the vessels to provide sufficient contact time with the vessel walls for vector to cross into the parenchyma. Animals that received a saline pre-

injection in place of mannitol showed no transgene expression in the brain. The highest virus doses resulted in dense staining that included both individual cell bodies and what we hypothesize to be areas of secreted protein in the extracellular space. Further, we found that TPP1 localizes to neuronal cells after IA delivery (Fig. 3i – 3l). Because the transgene product is a secreted protein we do not know if the colocalization of the immunofluorescence staining of TPP1 with neurons was due to innate production of TPP1 within these cells or neuronal uptake of protein secreted from neighboring cells. The results of quantitative staining analysis agreed with the qualitative results of Fig. 3 (Fig. 4).

The pattern of TPP1 expression we observed was closely correlated with the pattern of BBB disruption seen in our MRI studies (Fig. 5). The tissue sections from the whole ipsilateral hemisphere and the medial section of the contralateral hemisphere that corresponded with areas of signal enhancement on MRI showed extensive TPP1 expression, while lateral sections from the contralateral hemisphere displayed little to no TPP1 expression.

We then compared the biodistribution of TPP1 enzyme activity in multiple tissues after administration of 2.54×10^{11} gc of AAVrh.10CLN2 delivered via IA and intravenous (IV) routes with mannitol preinjection [19], to a treatment naïve control group (n = 3 animals per group). Tissue from the brain, heart, lung, spleen, liver, kidney, and quadriceps muscle were assessed for TPP1 enzymatic activity (Fig. 6). Significantly more TPP1 activity (greater than 6-fold) was detected in the brains of IA treated mice compared to the IV treated group (p = 0.003). Further, IA treated mice showed a trend towards decreased TPP1 activity in peripheral tissues versus IV treated mice (significant for skeletal muscle, p = 0.017), suggesting that the first pass uptake of IA delivered vector in the brain resulted in some sparing of peripheral tissues.

Minimum BBB disruption required for therapeutic efficacy

To determine the minimum degree of disruption required to render the BBB permeable to the AAVrh.10 vector, we injected mice with 3.18×10^{10} gc of AAVrh.10CLN2 after mannitol doses of 125 μ l, 250 μ l, 500 μ l, and 750 μ l (n = 3 animals per group). We found that there was a minimum level of disruption required for the AAV construct to cross the BBB (Fig. 7).

No transgene expression was observed for animals receiving the 125 μ l mannitol injection, and increased spatial distribution of staining was observed as the volume of injection was increased from 250 μ l to 750 μ l (Figs. 4, 7). Approximately 60% of treated survival animals that received the highest dose of mannitol exhibited no adverse response to the treatment. Animals were observed on a daily basis, and those that displayed lethargy, lack of appetite, or neurological deficit were sacrificed within 48 hours of the surgery. A detailed breakdown of observed morbidity versus mannitol dose for animals treated with AAVrh.10CLN2 is provided in Table 1. We observed little viral vector related toxicity in treated animals in the absence of high dose mannitol. Note that the numbers in the Table reflect the number of animals for whom morbidity was ultimately observed versus the total number of animals undergoing an identical procedure on the day of surgery. Since not all animals undergoing a procedure were harvested for staining, the total numbers in the Table are higher than the number of animals represented in Figure 4 (N=30 versus N=21).

Discussion

By performing DCE-MRI after BBB disruption we were able to quantify the impact of different IA mannitol injection conditions on the extent of disruption in different territories of the brain. We found that increasing the dose of mannitol resulted in increased BBB disruption. This was true for measurements over the whole hemisphere and in individual arterial territories (Fig. 2).

Many current clinical IA mannitol delivery protocols inject 25% wt/vol mannitol at the highest flow rate that exhibits no reflux on cerebral angiogram [23, 24]. In adults, this flow rate ranges from 3–12 ml/s and it is maintained for 30s. This protocol is similar to studies performed by Neuwelt *et al* in the late 1970s assessing BBB disruption by CT contrast extravasation in animal models [25]. These protocols have remained largely unchanged and do not account for the physicochemical properties of the delivered drug or the individual vascular anatomy of the targeted tissue. Understanding these parameters may be crucial for effective and efficient IA delivery of therapeutics as the action of mannitol on the BBB after IA delivery appears to be a complex system involving both pharmacokinetics and fluid dynamics.

We found that the total volume of mannitol delivered was the critical factor for BBB disruption. Our results suggest that current clinical protocols derived from studies of maximum obtainable injection flow rates might be insufficient for drug delivery to the CNS as widely different volumes of mannitol are delivered which may result in divergent degrees of BBB disruption between patients.

While the total volume of mannitol delivered was critical for sufficient BBB disruption, we found that changing the flow-rate of the mannitol injection had no significant effect (Fig. 2b). In other words, the increased contact time between the mannitol and the vessel wall did not result in increased BBB disruption, confirming that the 25–30 second injection duration commonly quoted in the literature is sufficient [8, 17, 26, 27]. However, the signal enhancement in the ACA territory observed for constant volume injections (Fig. 2b) may indicate that the contact time in the distal branches of the cerebrovascular tree is not long enough at the higher mannitol flow rates to obtain sufficient BBB disruption.

Our results also suggest possible reasons for inconsistent BBB disruption and pharmacologic results commonly reported in the literature. First, the volume of mannitol we injected to disrupt the whole mouse hemisphere is greater than what is typically delivered [17, 28]. Other smaller injection volumes may not penetrate deeply enough into the vascular tree to obtain sufficient BBB disruption in distal brain territories. Further, since Gd-DTPA is a small molecule, merely confirming its presence in brain parenchyma, either by histology or on non-quantitative MRI, is not a guarantee that the BBB has been disrupted enough to permit large therapeutics such as liposomes, nanoparticles, or viral vectors to pass from the blood to the parenchyma.

Our next goal was to assess if the degree of BBB disruption we observed would be sufficient to allow IA delivery of nanoscale therapeutic constructs to the brain. We chose to use AAVrh.10CLN2 for our proof of concept studies, as it is a relatively large biologic (23 nm

diameter) and successful IA delivery of this viral vector could lead to a potential minimally invasive single administration treatment for LINCL.

LINCL is a universally fatal, autosomal recessive pediatric neurodegenerative lysosomal storage disease. Mutations in the CLN2 gene result in a lack of production of tripeptidyl peptidase-1 (TPP1), a 46 kDa enzyme required by lysosomes for cleavage of N-terminal tripeptides from substrates. The dearth of TPP1 causes an accumulation of autofluorescent lipofuscin in lysosomes, leading to cell death. In humans, disease onset occurs at 2–4 years of age, and thereafter subjects suffer seizures, cognitive impairment, blindness, and loss of motor skills, with death occurring at ages 10–12 years.

Previous studies using AAV to produce TPP1 have shown that transgene production is robust in the long term, and that the volume of transfected tissue may increase with time [13]. This suggests that IA delivery of AAVrh.10CLN2 after BBB disruption may be an efficient way to obtain long-term and widespread transgene production in the brain after a single administration as a potential treatment for LINCL.

We also performed a biodistribution assay comparing the enzyme activity levels of TPP1 in targeted and peripheral tissues of naïve mice to those in mice given AAVrh.10CLN2 via IA injection with mannitol mediated BBB disruption and via IV injection 8 minutes after IV injection of mannitol (Fig. 6) [9]. The results show that IA delivery of vector after BBB disruption yields significantly more TPP1 activity (~6 fold higher) in the target brain tissue compared to IV treated and naïve mice. Further, we observed a trend toward reduced TPP1 activity in peripheral tissue for IA treated mice versus IV treated mice. This suggests that IA delivery of AAVrh.10 is a viable way to reduce systemic exposure while still achieving high levels of transgene expression and enzymatic activity in the target tissue. Future studies of this delivery method should incorporate assays to determine if the reduced systemic exposure between IA and IV treated groups has any statistically significant effects on organ function or toxicity due to the trend toward reduction of transgene expression observed in these peripheral tissues.

The potential systemic effect of TPP1 is theoretically advantageous in the case of a disease such as LINCL. While the primary manifestations are in the CNS there is an absence or deficiency of TPP1 throughout the body, therefore the effect of the disease is not confined to the brain. Similar expression in peripheral organs was observed when we injected the same vector directly into the CNS [12] of CLN2^{-/-} mice and no adverse effects were noted. In addition there is no known toxicity associated with over expression of TPP1 as has been seen in two separate toxicology studies we carried out in support of two clinical studies (one completed and one ongoing) where adeno-associated vector-mediated delivery of TPP1 was shown to be safe [29, 30].

The pattern of transgene expression was closely correlated with the BBB disruption results of our MRI studies. The degree of expression seen on immunoperoxidase staining decreased along the cerebrovascular tree, with the densest staining appearing in the AChA territory, followed by the MCA and ACA territories. This is similar to the enhancement trend seen in our MRI data (Fig. 2), where for a given mannitol injection condition the AChA territory is

the most disrupted, followed by the MCA territory, with the ACA territory being the least disrupted.

This trend was also seen in our mannitol dose escalation studies. We found that at the lowest 125 μ l mannitol dose we saw no transgene expression (Fig. 7a). At a mannitol dose of 250 μ l we observed TPP1 expression, but it was limited to the AChA territory only (Fig. 7b). As the mannitol dose was increased to 500 μ l and 750 μ l we saw increasing levels of cortical expression of TPP1, consistent with the increased BBB disruption observed in the distal vasculature in our MRI studies (Fig. 7c and 7d). As we have shown for AAVrh.10 there exists a cut-off point of minimal BBB disruption below which no transgene expression is observed. While mannitol is used routinely in the clinic for traumatic brain injury there are known dose dependent adverse physiological outcomes [31]. It is important to ascertain what IA mannitol dose is required to disrupt the BBB enough to allow transluminal passage of the therapeutic of interest. Depending on the physicochemical properties of the therapeutic construct in question more or less BBB disruption may be needed and the results we present herein may provide a method to determine optimal BBB agonist doses for other classes of therapy. In the case of LINCL, future survival studies of CLN2 $-/-$ mice would determine whether the maximum mannitol dose of 750 μ l that we used is necessary, or whether a reduced dose would be sufficient since we observed staining down to 250 μ l.

Conclusions

We showed that DCE-MRI is a powerful technique for quantifying the extent and pattern of BBB disruption after IA injection of a hyperosmotic mannitol solution. We found that the volume of injected mannitol is the most significant factor in BBB disruption, and that the degree of disruption is based on the order of arterial branching from the ICA. The flow rate of mannitol injection is not significant for determining disruption provided it is slow enough to allow sufficient contact time ($> \sim 30$ s) between the injected mannitol and the vessel wall in all targeted territories.

A careful combination of mannitol injection conditions with detailed knowledge of the targeted vascular anatomy may facilitate selective targeting of brain territories in animal models where traditional endovascular super selective IA (SSIA) techniques are impossible. Further, using this information in cases where SSIA is feasible, may allow for more effective and efficient IA delivery.

We also showed that IA gene therapy can provide widespread transgene expression after a single treatment. To our knowledge this is the first demonstration of successful IA gene therapy using an AAV vector in the mouse brain. Previous studies examining expression after intracerebral injection of the AAVrh10.TPP1 vector suggest that the levels we observed after IA delivery should be sufficient to arrest disease progression in knockout mouse models of LINCL.

Finally, we have shown that there exists a minimum threshold of BBB disruption necessary to obtain transgene expression in the central nervous system using AAV vectors. This threshold is likely different for each therapeutic but the methods used herein can be readily

applied to other diseases and drug constructs. Knowing these thresholds would allow for safer and more effective IA delivery strategies.

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Figure 1.

(a) Normalized MRI signal intensity versus time in the circular regions of interest supplied by the anterior choroidal artery (AChA), middle cerebral artery (MCA), and anterior cerebral artery (ACA) as shown in (b) for Gd-DTPA injected two minutes after BBB disruption by 750 μ l of 25 % wt/vol mannitol solution at a flow rate of 1000 μ l/min. (b) Baseline image taken before Gd-DTPA injection. (c) First pass of high concentration Gd-DTPA through the arterial system. The signal attenuation is caused by concentration dependent T_2 shortening effects. (d) After the injection Gd-DTPA extravasates into the parenchyma where the BBB has been disrupted. The resulting reduced concentration causes MRI signal enhancement via T_1 shortening that permits quantification of BBB disruption.

Figure 2.

The extent of BBB disruption (normalized signal enhancement over baseline) in the anterior choroidal artery (AChA), middle cerebral artery (MCA), and anterior cerebral artery (ACA) territories of the mouse brain for different mannitol injection conditions. (a) The effect of different mannitol injection volumes (0 µl, 125 µl, 250 µl, 500 µl, and 750 µl) at a constant flow rate (1000 µl/min). (b) The effect of different mannitol injection flow rates (250 µl/min, 500 µl/min, 800 µl/min, and 1000 µl/min) for a volume of 750 µl. Data is presented as the mean $\pm$ standard error of the mean ($n = 6$ for Gadolinium only treatment group; $n = 8$ for all other groups).

Figure 3.

Immunohistochemical staining of transgene production in mouse brain sections. Panels (a) – (h) show immunoperoxidase staining for TPP1. Note the increased staining density and distribution as virus dose is increased (panels (a) – (d)). (a) AAVrh.10CLN2 dose of 3.18 ± 10^{10} gc, (b) 6.35 ± 10^{10} gc, (c) 1.27 ± 10^{11} gc, (d) 2.54 ± 10^{11} gc/mouse, (e) 3.18 ± 10^{10} gc with saline rather than mannitol pre-injection (750 μl at 800 μl/min), (f) treatment naïve mouse, (g) close-up of cortex in the 2.54 ± 10^{11} gc treated mouse, (h) close-up of superior colliculus of 2.54 ± 10^{11} gc treated mouse showing patch of extracellular TPP1 protein. Panels (i) – (l) show immunofluorescent images demonstrating colocalization of transgene product with neurons in the cortex of a mouse treated with IA delivery of AAVrh.10CLN2. (i) Neuron specific stain. (j) TPP1 transgene product. (k) Cell nuclei (DAPI). (l) Merge of panels (i), (j), and (k).

Figure 4. Relative staining efficiency versus vector and mannitol dose. The data labels represent mannitol doses used at each vector dose. The inset shows the immunohistochemistry image of Fig. 3d after thresholding and binarization.

Figure 5.

Correlation between the pattern of enhancement observed on MRI with the distribution of transgene expression seen after BBB disruption using 750 μ l of mannitol and 2.54×10^{11} gc of AAVrh.10CLN2. TPP1 expression is seen in lateral and medial sections of the ipsilateral hemisphere (slices (a) and (b)). TPP1 staining is also present in the medial section from the contralateral hemisphere (slice (c)) due to the azygous origin of the ACA territory. However no transgene expression is seen in the lateral section of the contralateral hemisphere (slice (d)).

Figure 6.

Comparison between the biodistribution of TPP1 enzyme activity after delivery via either IV or IA routes with that of treatment naïve mice in 7 different tissues. IA and IV treated mice received the same mannitol (750 μ l) and virus (2.54×10^{11} gc AAVrh.10CLN2) doses. Data is presented as the mean $\pm$ standard error of the mean (n = 3 animals per group). * - significant with p-value of 0.003; ** - significant with p-value of 0.017.

Figure 7.

Immunoperoxidase staining showing the effect of increasing mannitol mediated BBB disruption on transgene expression. Each mouse received 3.18×10^{10} gc of AAVrh.10CLN2 after (a) 125 µl, (b) 250 µl, (c) 500 µl, or (d) 750 µl of mannitol at 800 µl/min. No transgene expression is seen in panel (a). At 250 µl, some TPP1 production is seen in deeper brain structures, and increasing degrees of staining are observed in cortical structures as the mannitol dose is increased to 500 µl and 750 µl (panels (c) and (d)).

Table 1

Morbidity versus mannitol dose for mice treated with AAVrh.10CLN2.

Mannitol	125 μ l	250 μ l	500 μ l	750 μ l	Total
Morbidity	1/4	0/4	0/4	6/18	7/30